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SK Verma
Central Institute for Cotton Research, Regional Station, Sirsa, Haryana, India

Babita
Department of Biotechnology, Chaudhary Devi Lal University, Sirsa, Haryana, India

Renu
Department of Biotechnology, Chaudhary Devi Lal University, Sirsa, Haryana, India

OP Tuteja
Central Institute for Cotton Research, Regional Station, Sirsa, Haryana, India

Corresponding Author:
SK Verma
Central Institute for Cotton Research, Regional Station, Sirsa, Haryana, India

Genetic diversity analysis of CGMS lines A, B and restorer lines in tetraploid cotton (*Gossypium hirsutum* L.) using RAPD markers

SK Verma, Babita, Renu and OP Tuteja

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Abstract
Random amplified polymorphic DNA (RAPD) analysis was employed in 5 CGMS lines, 5 B-lines and 8 restorer lines of tetraploid cotton (*Gossypium hirsutum*). The 20 primers selected generated 572 amplification products. Statistical analysis was carried out using NTSYS- pc software and a dendrogram was generated using Jaccard's similarity coefficients. The values for similarity coefficients ranged from 0.05 to 0.99. The cluster tree analysis showed the grouping of 18 genotypes in two major A and B groups at similarity coefficient of 0.35. The major group A consists of twelve genotypes and group B consists of remaining six genotypes. Cluster analysis showed clear-cut separation of the CGMS lines, B-lines and restorer lines. The primer OPO - 1 with 4 unique bands could distinguish K34007A and K34007B line and similarly Jhorar A and Jhorar B line. The primer OPO - 11 could distinguish restorer lines CIR 28 and CIR 38 scoring 4 and 3 unique bands respectively. The primers OPO - 12 and 20 also distinguished restorer lines CIR 28 and CIR 38. The primers OPO - 15 and 16 could distinguish restorer lines CIR 26 scoring 1 and 2 unique bands respectively.

Keywords: tetraploid cotton, CGMS lines A & B, restorer lines, genetic diversity, random amplified polymorphic DNA (RAPD)

Introduction
Cotton is the excellent and important source of world wide natural fiber. Besides, it is also an oil crop. Cotton (*Gossypium* spp.) is comprised of about 50 diploid and tetraploid species but world's cotton fiber is produced from four species, *G. arboreum* L (n = 13, A genome), *G. herbaceum* L (n = 13, A genome), *G. barbadense* L (n = 26, AD genome), and *G. hirsutum* L (n = 26, AD genome). About 90% of commercial cotton is obtained from *G.hirsutum*. Cotton is grown largely in China, USA, India, Pakistan etc. India is the largest cotton growing country in the world. The present lint yield in the country is 469kg/ha. It supplies 48% of the fiber to textile industries so it is called 'White Gold' in India.

For any crop improvement programme, analysis of genetic diversity is the first and foremost step followed by hybridization and exploitation of heterosis to produce high production of lint fiber in case of cotton. To have a reliable estimate of genetic relationships and genetic diversity a large number of polymorphic markers are required. Most of genetic diversity analysis studies in cotton have been carried out using morphological markers only. Now-a-days Polymerase Chain Reaction (PCR)-based molecular markers (Brar and Dhaliwal 1997 [1] and Lee 1999 [4]) have been developed into powerful tools to analyze genetic relationships and genetic diversity. Random amplified polymorphic DNA (RAPD) first reported by Williams *et al*. (1990) [9] is one of such tools. It is a powerful technique and its resolving power is several fold higher than morphological and isozyme markers and is much simpler and technically less demanding than other such techniques. The study by Iqbal *et al.* (1990) [3] clearly distinguishes *G. hirsutum* from those of *G. arboreum* with 98% of the primer found polymorphism.

RAPD markers have broad taxonomic applications. Since, they amplify large no. of DNA fragments per reaction. They don't require prior knowledge of DNA sequences. They are well suited for plant breeding application and DNA fingerprinting (Williams *et al.*, 1990) [9]. High resolution, polymorphism and reproducibility of RAPD-PCR assay in cotton provide effective system to breeders to assess genetic diversity and utilization in breeding programs. These DNA markers have wide application in the area of plant genome analysis and breeding (1994, Paterson *et al.*; 1991; Mohan *et al.*, 1997; Brar and Dhaliwal 1997; Lee 1999; Joshi *et al.*, 1999). Recently, RAPD analysis has been used for diversity analysis in a vast array of field crops.

It has been used for genetic diversity analysis of diploid and tetraploid species in cotton and in the present study the genetic diversity was studied in CGMS lines A, B and their restorers in upland cotton.

Materials and Methods

Plant material and DNA extraction

The present plant material comprised of 18 genotypes which include five CGMS (cytoplasmic genic male sterile) lines of *G. harknessii background* and their maintainer lines (fertile counter part of CGMS line) and eight restorer lines of *G.hirsutum*. The *Gossypium harknessii* Brandagee (D2-2) which is a diploid (2n=26) was used as female by Meyer (1975) to transfer *G. hirsutum* genome in the cytoplasm of *G. harknessii.* The resultant triploid was made hexaploid (2n=78) using colchicine. Male sterile tetraploid plants were recovered from cross between hexaploid and tetraploids. Five CGMS lines, Jhorar A, SH2379ckA, K34007A, PIL43ccA and F505A were developed at Central Institute for Cotton Research, Regional Station, Sirsa using IH 76 carrying *G. harknessii* cytoplasm through back cross breeding. The lines, CIR8, CIR10, CIR12, CIR23, CIR26, CIR28, CIR38 and CIR70 selected from the germplasm collections were identified as restorer lines based on the performance of F_1 when crossed with CGMS lines. The list of selected genotypes is given in table 1. Genomic DNA from each genotype was isolated from bulked young leaf samples using modified CTAB (cetyl trimethyl ammonium bromide) of Saghai-Maroof *et al.* (1984). DNA was checked for its quality and quantity by 1% agarose gel electrophoresis and by spectrophotometer.

Table 1: List of CGMS lines A, B and restorer lines of G. *hirsutum*

Sr. No.	Genotypes	Lines
1.	Jhorar A	A-Lines (CGMS)
2.	SH2379ckA	A-Lines (CGMS)
3.	K34007A	A-Lines (CGMS)
4.	PIL43ccA	A-Lines (CGMS)
5.	F505A	A-Lines (CGMS)
6.	Jhorar B	B-Lines (Fertile)
7.	SH2379ckB	B-Lines (Fertile)
8.	K34007B	B-Lines (Fertile)
9.	PIL43ccB	B-Lines (Fertile)
10.	F505B	B-Lines (Fertile)
11.	CIR8	Restorer-Lines
12.	CIR10	Restorer-Lines
13.	CIR12	Restorer-Lines
14.	CIR23	Restorer-Lines
15.	CIR26	Restorer-Lines
16.	CIR28	Restorer-Lines
17.	CIR38	Restorer-Lines
18.	CIR70	Restorer-Lines

RAPD Marker Analysis

A total of 20 RAPD (10-base oligonucleotide primers, Operon Technologies, Inc., U.S.A.) primers were used for PCR amplification. PCR amplifications were performed by using JH-BIO cooled gradient palm-cycler (Corbett Research, Australia Ltd). The 25 µl PCR reaction mixture contained 10X PCR buffer (without $MgCl_2$), 25mM $MgCl_2$, 2.5mM dNTPs, and 10 mM of primer, 5 unit of taq DNA polymerase and 2 µl (50ng) DNA templates. PCR amplifications using different primers were performed at 38 °C annealing temperatures. PCR amplifications were performed with hot start at 94 °C for 5 min followed by 45 cycles of denaturation 94 °C for 1 min, annealing at 36 °C for 1 min and extension at 72 °C for 2 min. Final extension was carried out for 7 min at 72 °C before cooling at 4 °C for 10 min. The amplified DNA fragments were separated by 1% (w/v) agarose gel electrophoresis in Tris-Borate EDTA buffer. The size (in nucleotide base pairs) of the most intensely amplified band was determined based on its migration relative to molecular weight size markers (1000 bp ladder). Samples were loaded in the wells and electrophoresis was carried out at constant voltage of 65 volt at 400 milli ampere for one and half hour. The DNA bands were visualized by staining the gels with ethidium bromide and photographed under UV light using gel documentation system (Alpha Innotech Corporation, California, USA).

Band Scoring and data analysis

Polymorphic bands for RAPD analysis were scored based on the presence or absence of each band for each cotton variety. Only clear and unambiguous bands were scored. The frequency of RAPD polymorphism among 18 genotypes of cotton for each marker was calculated based on the presence (taken as ´1') or absence (taken as ´0') of common bands (Ghosh *et al*., 1997) [2]. Multani & Lyon (1995) [6] generated RAPD markers with 30 random primers and found that *G. barbadense* accession can be easily distinguished from *G. hirsutum* variety by 104 markers. The genetic association between varieties was evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on proportion of shared bands produced by primers. Similarity matrix was generated using 'simqual' sub programme of software NTSYS-PC (Numerical Taxonomy System software, version 2.1). Jaccard similarity coefficients were used for cluster analysis of genotypes using 'SAHN' subprogram and dendrogram was built based on the UPGMA (unweighted pair group method with arithmetic average). Principal coordinate analysis (NTSYS-pc) was performed to generate a two-dimensional representation of genetic relationship among 18 cotton genotypes involving CGMS and restorer lines.

Result and Discussion

RAPD Banding Profile and Polymorphism

A total of 572 clear bands were produced using 20 RAPD markers in 18 genotypes involving CGMS and restorer lines (Table 2). The number of amplified DNA fragments (bands) varied from eight (RAPD primer OPO -10) to 65 (RAPD primer OPO -12) with average number of 28.6 bands per primer. The maximum number of polymorphic bands (65) was obtained for RAPD primer OPO -12. Hundred per cent polymorphism was obtained for all the primers screened. Each of 20 RAPD primers generated high level of polymorphism and could differentiate between majority of the CGMS lines and the fertile counterparts and restorer lines except some of the closely-related lines as shown in Fig. 1.

Putative Genotypes/Group Specific Bands

Out of the 572 bands, 124 were identified as unique bands which were present exclusively in CGMS line A or B (Table 3). These unique bands were produced using 20 RAPD primers with overall frequency of 6.2 unique bands per primer. Maximum number (8) of unique bands was scored with OPO - 16 and 12. The maximum number of unique bands (8) was recorded in CGMS lines PIL 43ccB, F 505B and K34007A. The primer OPO - 1 with 4 unique bands could distinguish K34007A and K34007 B line and similarly Jhorar B and Jhorar A line. The CGMS line K34007A was distinguished by most of the primers screened. The CGMS

line PIL 43ccB was distinguished by six primers while CGMS line F 505B and Jhorar B by five primers. The CGMS line SH2379ckA and SH2379ckB were distinguished by one primer each.

In case of restorer lines, out of 572 bands, 37 were identified as unique bands which were present exclusively in only one of the restorer lines (Table 4). These unique bands were produced by 8 primers with overall frequency of 4.63 unique bands per primer. Maximum number (7) of unique bands was scored with OPO -5. Out of the 8 restorer lines, five restorer lines CIR 8, CIR 10, CIR 12, CIR 23 and CIR 70 did not have any unique bands. The maximum number of unique bands (7) was recorded in restorer line CIR 38. The primer OPO - 11 could distinguish restorer lines CIR 28 and CIR 38 scoring 4 and 3 unique bands respectively. The primers OPO - 12 and 20 also distinguished restorer lines CIR 28 and CIR 38. The primers OPO - 15 and 16 could distinguish restorer lines CIR 26 scoring 1 and 2 unique bands respectively.

Table 2: Total number of polymorphic bands in CGMS lines A, B and restorer lines

S. No.	Primer no.	Sequences (5'-3')	Total no. of bands	Average number of bands per genotypes
1.	OPO- 1	5'-GGC ACG TAAG- 3'	21	1.16
2.	OPO-2	5'- ACG TAG CGTC-3'	25	1.38
3.	OPO-3	5'-CTG TTG CTAG- 3'	36	2.00
4.	OPO-4	5'-AAG TCC GCTC-3'	34	1.88
5.	OPO-5	5'-CCC AGT CACT-3'	38	2.11
6.	OPO-6	5'-CCA CGG GAAG-3'	32	1.77
7.	OPO-7	5'-CAG CAC TGAC-3'	32	1.77
8.	OPO-8	5'-CCT CCA GTGT-3'	18	1.00
9.	OPO-9	5'-TCC CAC GCAA-3'	22	1.22
10	OPO-10	5'-TCA GAG CGCC-3'	46	2.55
11.	OPO-11	5'-GAC AGG AGGT-3'	21	1.16
12.	OPO-12	5'-CAG TGC TGTC-3'	65	3.61
13.	OPO-13	5'-GTC AGA GTCC-3'	16	0.88
14.	OPO-14	5'-AGC ATG GCTC-3'	17	0.94
15.	OPO-15	5'-TGG CGT CCTT-3'	30	1.66
16.	OPO-16	5'-TCG GCG GTTC-3'	46	2.55
17.	OPO-17	5'-GGC TTA TGCC-3'	8	0.44
18.	OPO-18	5'-CTC GCT ATCC-3'	11	0.61
19.	OPO-19	5'-GGT GCA CGTT-3'	20	1.11
20.	OPO-20	5'-ACA CAC GCTG-3'	34	1.88
Total			572	
Mean			28.6	

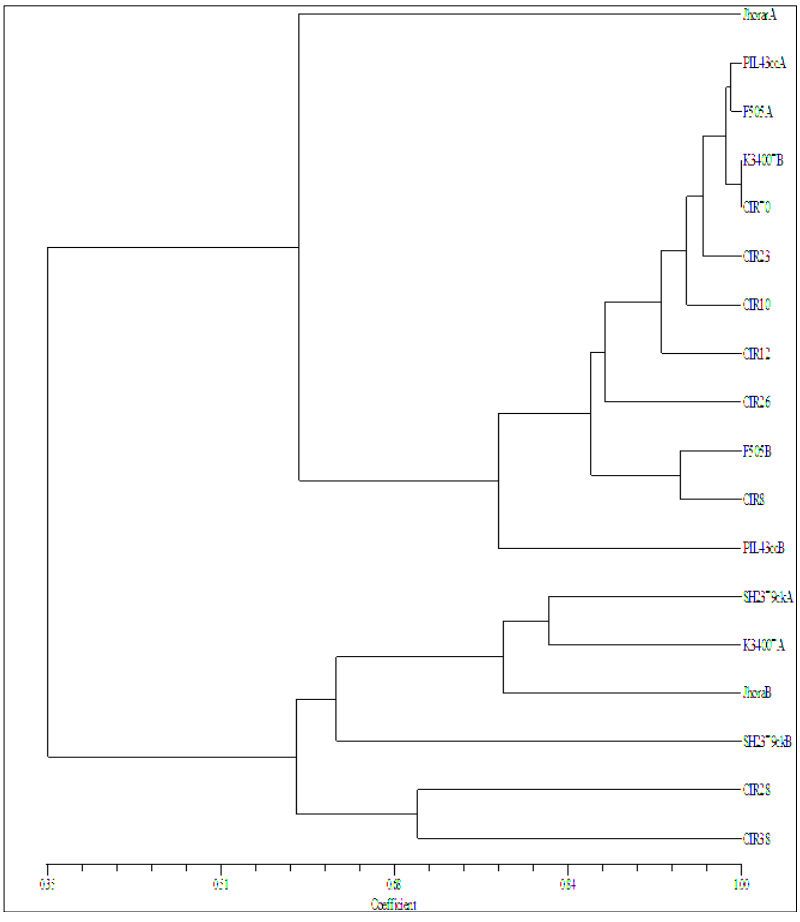

Fig 1: Dendrogram illustrating genetic relationship among CGMS, B and restorer lines in *G. hirsutum*

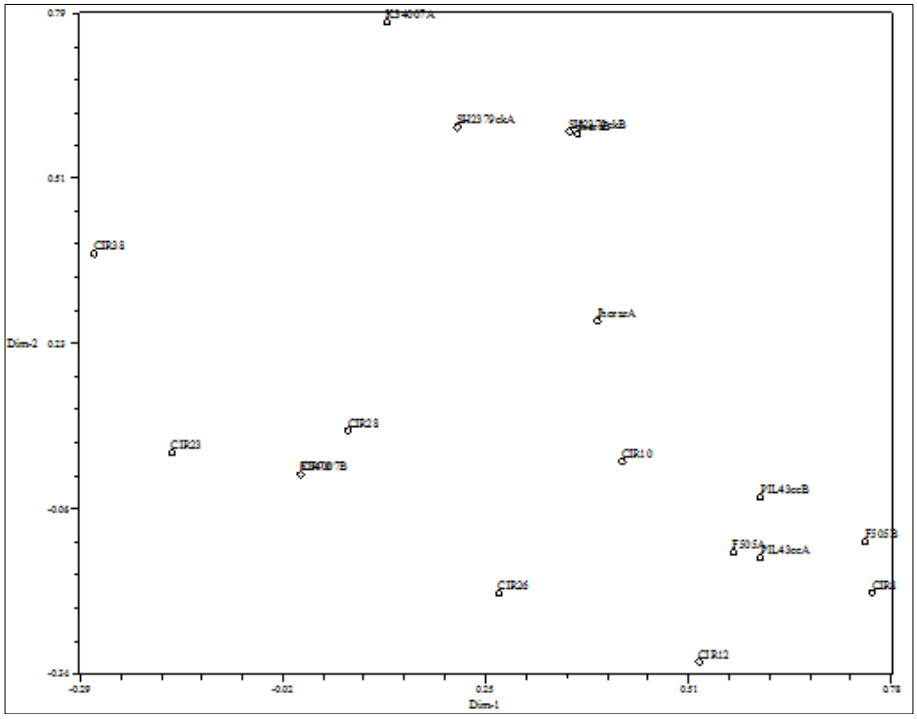

Fig 2: Two dimensional plot of genetic diversity among 18 cotton genotypes as revealed by the PCA (NTSYS-pc) of RAPD marker

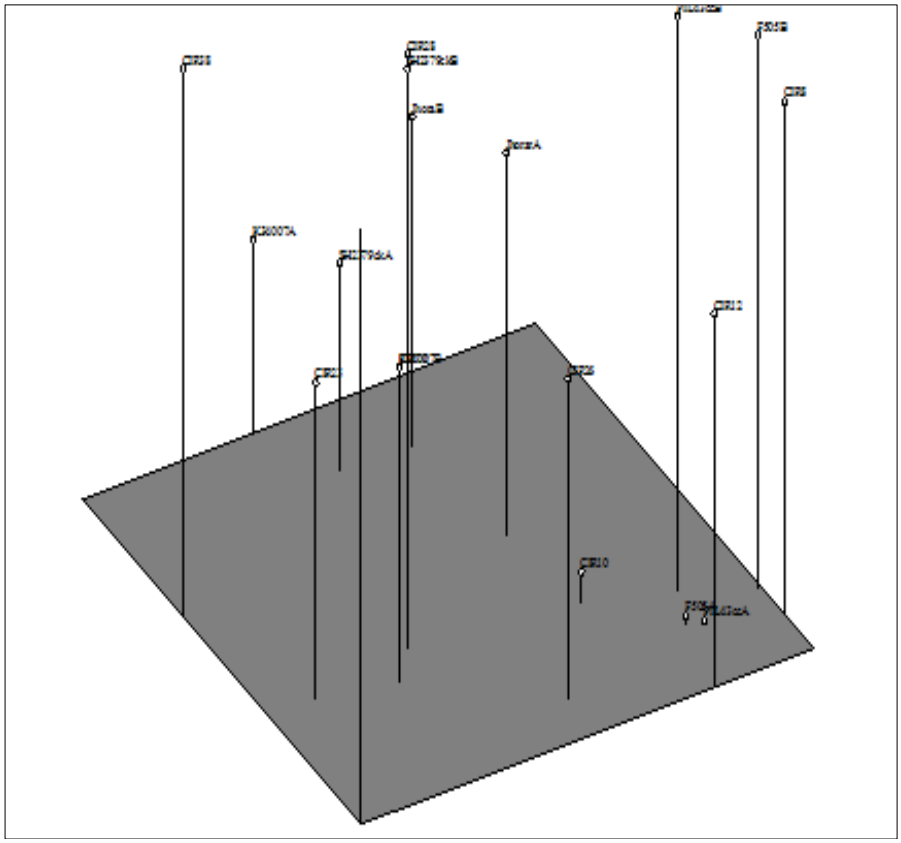

Fig 3: Three dimensional plot of genetic diversity among 18 cotton genotypes as revealed by the PCA (NTSYS-pc) of RAPD marker

The groups identified by PCA were very similar to those generated by UPGMA based dendrogramas shown in Fig 2 and Fig 3.

Similarity Cluster of Cotton Genotypes (CGMS line A, B and restorer lines)

The Jaccard's similarity coefficient as calculated using Simqal' subprogram of NTYSYS-pc software revealed maximum similarity (0.991) between the PIL43ccA and F505A (CGMS line). Jaccard's similarity coefficient between the various genotypes was used to produced dendrogram (cluster tree analysis) and a two dimensional scale diagram (PCA analysis) which clearly explain the relationship among the 18 cotton CGMS lines A, B and restorer lines. The value of ranged from 0.35 to 1.00. The cluster tree analysis showed the grouping of 18 genotypes in two major A and B groups at similarity coefficient of 0.35. The major group A consists of twelve genotypes and group B consists of remaining six genotypes. The major group A are further divided in to sub groups A1 and A2. The sub group A1 and A2 showed the

similarity coefficient 0.585. GroupA1 consist only one genotype 'Jhorar A' CGMS line. The subgroup A2 consists of F 505B and CIR 8 genotypes with the similarity coefficient 0.942. There is no distinction between the two genotypes K 34007B and CIR70. The major group B also further divided in to sub groups B1 and B2 with similarity coefficient 0.089. The subgroup B1consist of five genotypes having the similarity coefficient 0.62 and B2 consist of two restorer lines CIR28 and CIR 38 with similarity coefficient 0.70. Similarly Punitha *et al.* (2003) evaluated 15 cotton genotypes for polymorphic analysis using 32 different 10-mer primers of arbitrary sequences on a collection of 1l coloured cotton and four white linted genotypes of *Gossypium hirsutum*. Cluster analysis showed clear-cut separation of the coloured and white linted genotypes and thus formed three clusters (I, II and III). Likewise Rana *et al.* (2002) [8] employed Random Amplified Polymorphic DNA (RAPD) analysis in commercially released 18 cultivars of diploid cotton belonging to two species i.e. *Gossypium arboreum* and *Gossypium herbaceum*. All primers produced polymorphic amplification products and the values for similarity coefficients ranged from 0.50 to 0.99. Cluster analysis showed clear-cut separation of the genotypes of the two species.

Table 3: Distribution of unique bands present in CGMS lines A and B-lines of *G. hirsutum*

Primer No.	No. of unique bands present in only one line	CGMS or B-line having unique bands
OPO- 1	4	K 34007A, Jhorar B
OPO-2	4 (1)	K 34007A, (F 505A)
OPO-3	7	Jhorar B, K 34007A
OPO-4	5	K 34007A
OPO-5	9	K 34007A
OPO-6	5	K 34007A
OPO-7	6 (5), 3	Jhorar B, (K 34007A), PIL 43ccB
OPO-8	4	SH 2379ckA
OPO-9	3	K 34007A, PIL 43ccB
OPO-10	5	K 34007A
OPO-11	5(4)	Jhorar B, K 34007A (SH 2379ckB)
OPO-12	6(8)	K 34007A, (PIL 43ccB, F 505B)
OPO-13	2	K 34007A
OPO-14	3	K 34007A
OPO-15	3(2)	K 34007A, (PIL 43ccB, F 505B)
OPO-16	8 (2)	K 34007A, (PIL 43ccB, F 505B)
OPO-17	3 (2)	K 34007A, (Jhorar B)
OPO-18	2	K 34007A,
OPO-19	4	K 34007A,
OPO-20	5 (3), 1	K 34007A, (PIL 43ccB), F 505B
Total	124	
Average	6.2	

Table 4: Distribution of unique bands present in restorer-lines of *G. hirsutum*

Primer No.	No. of unique bands present in only one line	Restorer-line having unique bands
OPO -5	7	CIR 38
OPO -9	1	CIR 28
OPO -11	4	CIR 28
OPO -11	3	CIR 38
OPO -12	6	CIR 28
OPO -12	3	CIR 38
OPO -15	1	CIR 26
OPO -16	2	CIR 26
OPO -19	3	CIR 38
OPO -20	3	CIR 28
OPO -20	4	CIR 38
Total	37	
Average	4.63	

Study of DNA using molecular markers aids breeders to select appropriate genotype and also give opportunities to study chromosome evolution and to exploit genetic resources of *Gossypium* for its improvement. Molecular markers have helped in getting valuable genetic information about diversity and variation among genotypes. A lot of information about gene identification and map placement has increased the knowledge of desired agronomic traits and help in various crop improvement programmes. Molecular markers based on PCR are useful compliment to morphological and physiological characterization of cultivar because they are plentiful, independent of developmental stages of tissue or environmental effects and allow cultivar identification early in plant development. They are the tools to analyze genetic diversity. PCR based analysis give new dimension to concerted efforts of breeding and markers aided selection that reduce time for improvement of new varieties.

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